Comparative Analysis of Extracellular and Intracellular Proteomes of *Listeria monocytogenes* Strains Reveals a Correlation between Protein Expression and Serovar[▽][†]

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Listeria monocytogenes, the etiologic agent of listeriosis, remains a serious public health concern, with its frequent occurrence in food environments coupled with a high mortality rate. Among the 13 serovars, human listeriosis is mostly associated with the serovar 4b, 1/2b, and 1/2a strains. To investigate the diversity of *L. monocytogenes*, the intracellular and extracellular proteins of 12 strains were analyzed by two-dimensional gel electrophoresis. These strains had different origins, belonged to different serovars (4b, 1/2a, and 1/2b), and presented with different levels of virulence in chicken embryos. The clustering of the strains in two groups based on proteomic patterns is in agreement with the *L. monocytogenes* phylogenetic lineages. Statistical analysis did not allow for identification of proteins specific to the isolate origin or the virulence level of the strains, but 26 and 21 protein spots were shown to be significantly overexpressed and underexpressed, respectively, in the six strains of serovar 1/2a (lineage II) compared to strains of serovar 1/2b or 4b. Moreover, a penicillin-binding protein was specific for serovar 1/2b and two protein spots identified as a serine protease were specific to serovar 4b. These protein spots, identified through peptide mass fingerprinting using matrix-assisted laser desorption ionization–time of flight mass spectrometry, were essentially found in the extracellular proteome and may have uses as potential markers for serotyping and risk analysis.

Listeria monocytogenes is the causative agent of listeriosis, a severe disease that mainly affects pregnant women, newborn children, the elderly, and immunocompromised persons. The clinical features include severe gastroenteritis, mother-to-child infection, and central nervous system infections (37). Listeriosis is a relatively rare disease compared to other food-borne illnesses, with an incidence of between 2 and 10 reported cases per million people per year. However, because of its high mortality rate (20 to 30%), listeriosis ranks among the most frequent causes of death due to food-borne illness since it ranks second just after salmonellosis in the United States and France (21, 36). *L. monocytogenes* is problematic in the food industry since, apart from its ability to form biofilms, it can tolerate a wide range of temperatures, salt concentrations, and pHs. Despite significant efforts worldwide by research organizations and the food industry to reduce the incidence of listeriosis, this ubiquitous gram-positive pathogenic bacterium remains a critical threat to the safety of the food supply.

The infectious cycle of *L. monocytogenes* is now well known (37). First, internalin A (InlA) and InlB permit adhesion to the surface of the eukaryote cell and penetration into the host cell via phagocytosis. Then, bacteria escape the phagocytic vacuole by the production of a hemolysin called listeriolysin O (LLO) in combination with phospholipase A (PlcA) and PlcB. Once free from the vacuole, the bacteria multiply and become motile, gaining propulsion from the rapid polar polymerization of host actin performed by the bacterial protein ActA. The bacteria can spread to neighboring cells by coming into contact with the membrane and pushing it, which leads to the formation of pseudopods, which are engulfed by phagocytosis. That results in the formation of a secondary phagosome delimited by a double membrane and the beginning of another cycle (37).

Although these known virulence factors are present in the 13 different serovars of *L. monocytogenes* (10), only 3 serovars, i.e., 4b, 1/2a, and 1/2b, are responsible for 96% of the human listeriosis cases (49%, 27%, and 20%, respectively, in France between 2001 and 2003) (16). Whereas serogroup 1/2 strains are often found in the environment, the serovar 4b strains are isolated with lower frequency from food or from the industrial environment. For example, in dried sausage processing plants, the majority of the *L. monocytogenes* isolates were of serogroup 1/2 (49.5% of serovar 1/2a, 13.0% of serovar 1/2b, and 19.0% of serovar 1/2c) whereas only 8.0% of the isolates were of serovar 4b (35). Similar observations on *Listeria* strains isolated from soft and semisoft cheeses were made (20). Consequently, it was suggested that serovar 4b strains have a higher epidemic potential than serogroup 1/2 strains and that the

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TABLE 1. Strains of *L. monocytogenes* used for this study[a]

Strain	Serovar	Virulence	Information	Reference or source (reference[s])[b]
CLIP80459	4b	A	Human, epidemic, France, 1999	9
CLIP90602	1/2b	A	Human, epidemic, France	33
CLIP92347	1/2a	A	Human, epidemic, France	This study
EGDe	1/2a	A	Rabbit outbreak, England, 1924	14
CLIP93667	4b	A	Healthy 3-year-old child, France, 1992	H2 (27, 28, 33)
CLIP93679	4b	A	Healthy 39-year-old man, France, 1991	H12 (28)
CLIP93672	1/2b	B	Healthy 7-year-old child, France, 1994	H28 (28)
CLIP93677	1/2a	B	Healthy carrier, France	H38 (28)
CLIP93665	4b	A	Milk plant, France	33
CLIP93649	1/2a	A	Milk plant, France	33
CLIP93663	1/2a	B	Milk plant, France	This study
CLIP93657	1/2a	B	Milk plant, France	This study

[a] The levels of virulence of the 12 strains in 14-day-old chick embryos were assessed as previously described (28). Virulence A means infection of chick embryos resulted in 100% mortality within 3 days. Virulence B means infection of chick embryos resulted in 80% mortality in more than 3 days. CLIP stands for Collection *Listeria* Institut Pasteur.

[b] H2, H12, H28, and H38, strains referenced by Olier et al. (28).

serogroup 1/2 strains are better adapted to survive in food plants than serovar 4b strains.

The current revolution in microbial investigations due to the sequencing of complete genomes has revealed new insights into the genetic structures of a number of bacterial species. In *L. monocytogenes*, whole-genome comparisons of two strains of serovar 4b and two strains of serovar 1/2a (25) showed that 51 and 83 genes were, respectively, specific to serovar 4b and serovar 1/2a. Genomic comparison of 93 *L. monocytogenes* strains using DNA microarrays revealed distribution into three distinct lineages, with serovars 4b, 1/2b, and 3b found predominantly in lineage I and with serovars 1/2a, 1/2c, and 3c found predominantly in lineage II (10), which corroborated previous molecular subtyping data (5, 17, 26, 39).

Although a large number of discriminatory techniques to subtype *L. monocytogenes* exist, none of them are able to predict the ability of a strain to cause disease. It should also be stressed that there are discrepancies between data at the gene level (i.e., coded information) and data at the protein level (functional entity) since gene expression can be regulated at different levels and can lead to different phenotypes. Thus, the comparison of the protein contents of different strains could bring new insight into the survival mechanisms of this bacterium in the environment and food as well as into bacterial virulence. Proteomic analysis of cellular proteins of *L. monocytogenes* strains of different serovars indicated large intraspecies variability, as only 75 protein spots were found to be common to all strains (15). This analysis permitted for the first time apprehension of the biodiversity of *L. monocytogenes* by a proteomic approach and allowed the classification of strains into two clusters identical to the lineages previously found by molecular biology techniques. This investigation, though, focused on whole cellular protein contents and did not take into account the nature of proteins with different levels of expression. In order to better understand the differences between strains of *L. monocytogenes*, which could have a role in niche adaptation or in virulence, we compared the extracellular and the intracellular protein patterns of 12 strains of different ser-

ovars that were isolated from different environments and exhibited different levels of virulence.

MATERIALS AND METHODS

Strains and bacterial culture. The *L. monocytogenes* strains provided by the Institut Pasteur (Paris) are listed in Table 1. After two precultures in brain heart infusion, cells in stationary phase were transferred to MCDB202, a chemically defined medium (CryoBiosystem, L'Aigle, France), supplemented with 0.36% (wt/vol) glucose and adjusted to pH 7.3. The MCDB202 medium was supplemented before use with 0.1% (vol/vol) trace elements and 1% (vol/vol) of a solution of 10% (wt/vol) yeast nitrogen base. The 250-ml cultures were adjusted to an optical density at 600 nm (OD_{600}) of 0.1 and incubated at 37°C with shaking (150 rpm) until the late exponential phase (optical density at 600 nm = 0.9; 3- to 4-h cultures). The cells were harvested by centrifugation (15 min, 7,500 × g, 4°C).

Extraction of cytosoluble proteins. Cell pellets were washed twice with 1 ml TE buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM $MgCl_2$) and then resuspended in TE buffer, pH 9.0, and stored at −20°C until their treatment. Cells were sonicated with a Vibracell sonifier (Bioblock Scientific, Ilkirch, France) 10 times for 2 min at power level 5 and 50% of the active cycle. The samples were then treated with 1% DNase I and 1% RNase A for 30 min at room temperature. A solution containing final concentrations of 4 M urea, 2 M thiourea, 2% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, and 2 mM tributylphosphine was added, and the incubation was allowed to continue for 30 min on ice with intermittent agitation. The cells were eliminated by centrifugation (10 min, 4,500 × g, 4°C), and the supernatant was ultracentrifuged (30 min, 200,000 × g, 4°C). The supernatant was collected, and proteins were quantified by the method of Bradford (4). Bovine serum albumin was used as a standard. The protein samples were precipitated with 3 volumes of cold acetone at −20°C overnight and then pelleted by centrifugation (13,000 × g, 40 min). Pellets were resuspended in isoelectric focusing (IEF) buffer (5 M urea, 2 M thiourea, 2% [wt/vol] CHAPS, and 10 mM Tris-HCl in 50% [vol/vol] trifluoroethanol and a trace of bromophenol blue) at a concentration of 5 μg μl^{-1} proteins and stored at −20°C.

Precipitation of extracellular proteins. The supernatants of the cultures were filtered (0.2 μm), and 0.2 mM phenylmethylsulfonyl fluoride was added to inhibit protease activity. Na deoxycholate (0.2 mg ml^{-1}) was added to the solution, and the solution was incubated for 30 min on ice. Na deoxycholate supports protein precipitation, which was carried out by the addition of 10% (wt/vol) trichloroacetic acid and incubation overnight at 4°C. After centrifugation (20,300 × g, 30 min, 4°C), the precipitate was washed with ice-cold acetone and solubilized in IEF buffer.

Two-dimensional electrophoresis (2-DE). For IEF, precast immobilized pH gradient (IPG) strips with a pH 3 to 10 nonlinear gradient (extracellular proteins) or pH 4 to 7 linear gradient (intracellular proteins) were passively rehydrated for 17.5 h in a reswelling tray with 400 μl of IEF buffer containing 0.3%

(vol/vol) ampholytes for pH 3 to 10 (extracellular proteins) or 0.15% (vol/vol) ampholytes for pH 4 to 6 and 0.15% (vol/vol) ampholytes for pH 5 to 7 (intracellular proteins), 2 mM TBP, and 50 μg of proteins. The proteins were first subjected to IEF for a total of 66,450 V h (7 h at 50 V, 2 h at 200 V, linear gradient to 1,000 V in 2 h, 1 h at 1,000 V, linear gradient to 8,000 V in 5 h, and 8,000 V till the end). The strips were equilibrated twice for 15 min in an equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% [wt/vol] sodium dodecyl sulfate [SDS], 30% [vol/vol] glycerol) containing 2 mM TBP for the first step and 2.5% (wt/vol) iodoacetamide and traces of bromophenol blue for the second step. The second-dimension electrophoresis (SDS-polyacrylamide gel electrophoresis) was carried out with 12.5% acrylamide gel in a Multicell Protean II XL system (Bio-Rad, Marnes-la-Coquette, France). The gels were silver stained according to the method described by Rabilloud (29).

Image analysis. In order to limit variations due to manipulation, two extractions from two different cultures were carried out and at least three 2-DE gels per sample were run per subproteome. Stained 2-DE gels were scanned by a GS-800 imaging densitometer (Bio-Rad, Marnes-la-Coquette, France), and image analysis was performed using ImageMaster 2D Platinum software, version 5.0 (GE Healthcare, Uppsala, Sweden). Under these conditions, very similar protein patterns were obtained for a given strain. For each strain, a scatter plot was performed with all the gels. The scatter plot in ImageMaster 2D Platinum gives information about the relationship between the spot values from two 2-DE gels by searching for the linear dependence between the values from one gel and the values from another gel. For each subproteome, the five most reproducible gels from two cultures for a strain were selected and included for image analysis. These selected gels were then matched with the reference gel, and a number was assigned to each group of spots matched. The reference gel was generated from a mixture of equivalent quantities of proteins of the 12 strains, in order to match the maximum number of spots. Relative spot volume, i.e., digitized staining intensity integrated over the area (volume) of the individual spot divided by the sum of volume of all spots and multiplied by 100 was used for spot quantification. A matrix was obtained with the % volume of 600 spots and 599 spots for the 60 gels of the intracellular and extracellular proteomes, respectively.

Cluster analysis. For cluster analysis, only reliable protein spots were taken into account. A spot was considered reliable when it was present or absent in at least four gels out of the five gels per strain. When the spot was present in four out of five gels per strain, the absent value was replaced by the mean of the four other values. When the protein spot was reliably absent, the absent values were replaced by the lowest value for the gel. All of the values were transformed in $\log_2$ ratio % volume (22). In other words, each protein spot volume was divided by the average value of all the existing values for this protein spot in all the gels and then subjected to the base 2 logarithmic transformation. The matrix thus obtained contained 219 and 159 reliable protein spots for the intracellular and extracellular proteomes, respectively. The hierarchical clustering analysis was carried out with PermutMatrix (6) (http://www.lirmm.fr/~caraux/PermutMatrix/). Clustering results were calculated using an algorithm resulting from the combination of Ward's aggregation method and the Pearson-based distance metrics for the clustering algorithm. A preliminary study has shown that these parameters were the ones which gave the most reliable clusters (22). PermutMatrix allows for clustering result visualization with a dendrogram of the samples and a dendrogram of the protein spots.

Calculation of ERV. For the two-reliable-protein-spot matrix, the estimated relative variances (ERV) of the factors "virulence," "origin," and "serovar" were calculated (ERV = [sum of squares of the differences for the studied factor/sum of squares of the differences of the total variations] × 100). The higher the value, the higher is the dependence of the expression of the spot on the studied factor. We then computed the Fisher value and the corresponding *p* value (indicating the significance of the variations) for each spot for the three factors, virulence, origin, and serovar, according to three independent analysis of variance models ($x = \mu + F_i + \varepsilon$, with x = spot value, μ = mean effect, F_i = considered factor, and ε = residual variation). In order to discriminate the levels of the factors origin and serovar, two factorial discriminant analyses (FDA) were carried out using significant spots ($P < 40\%$ and 80%, respectively) with the greatest ERV. The probability of introduction and rejection of the variables (spots) was fixed at a *P* value of $<10^{-3}$. To discriminate virulence levels A and B of the strains, a stepwise FDA was performed to select a subset of independent variables among the 159 and 219 reliable spots of the extracellular and intracellular proteomes, respectively. Computations were carried out with the STATISTICA software, version 6.1 (StatSoft, France).

Identification of proteins by mass spectrometry. Semipreparative 2-DE gels containing 600 μg of proteins were run for protein spot identification. Protein spots were stained with colloidal Coomassie blue, and spots of interest were excised in the gel and subjected to the following treatments. First, the spots were washed in 25 mM ammonium bicarbonate–5% acetonitrile for 30 min and twice in 25 mM ammonium bicarbonate–50% acetonitrile for 30 min each. The spots were then dehydrated with 100% acetonitrile. The dried gels were reswelled in 25 mM ammonium bicarbonate containing 20 ng μl⁻¹ trypsin. Digestion was performed at 37°C for at least 5 h. The resulting peptides were extracted with 100% acetonitrile. After 15 min at 37°C, 1 μl of each sample and 1 μl saturated α-cyano-4-hydroxycinnamic acid matrix were mixed onto the matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) target. Positive-ion MALDI mass spectra were recorded in the reflectron mode of a MALDI-TOF mass spectrometer (Voyager DE-Pro; PerSeptive BioSystems, Farmingham, MA) using Voyager software for data collection and analysis. The mass spectrometer was calibrated with a standard peptide solution (Proteomix; LaserBio Labs, Sophia-Antipolis, France). Internal calibration of samples was achieved by using trypsin autolysis peptides. Monoisotopic peptide masses were assigned and used for NCBI database searches with the Mascot software. The following parameters were considered for the searches: a maximum fragment ion mass tolerance of ±25 ppm, possible modification of cysteines by carbamidomethylation, and oxidation of methionine.

RESULTS

General features of proteome patterns. Serovars, origins, and virulence levels of the 12 strains analyzed in this study are summarized in Table 1. The *L. monocytogenes* strains investigated belong to one of the three serovars responsible for 96% of the human listeriosis cases, i.e., 4b, 1/2a, or 1/2b. These strains originated either from clinical samples, asymptomatic carriage, or food plant environments. Two levels of virulence, i.e., A and B, were considered for each *L. monocytogenes* isolate and were based on the mortality of infected chick embryos (28). Virulence level A means infection of chick embryos resulted in 100% mortality within 3 days. It can be noticed that all the strains of serovar 4b belonged to this category. Strains were of virulence level B when they resulted in 80% mortality in more than 3 days (Table 1). No strain was found to be avirulent in this study. Growth curves of all strains were also compared but did not show any significant differences (data not shown).

Following growth at 37°C until the late exponential phase, the intracellular and extracellular protein fractions were isolated for comparative proteome analyses. Because the majority of intracellular protein spots of *L. monocytogenes* fall between pH 4 and 7 (11), linear pH 4 to 7 IPGs were used for this subproteome. As some abundant extracellular proteins were present in the basic zone, we decided to use nonlinear pH 3 to 10 IPGs to study the extracellular proteome. For each subproteome, 2-DE was carried out with a mixture of equivalent quantities of proteins of the 12 strains and the gel was used as a reference gel for the image analysis to match the maximum number of spots. These 2-DE reference gels were further used to visualize all the identified protein spots (Fig. 1).

As indicated by protein patterns on the 2-DE gels from intracellular extracts, many spots were common to the 12 *L. monocytogenes* strains. In contrast, the extracellular proteomes revealed more-pronounced differences in the expression patterns. Nevertheless, some spots were specific to some strains in both subproteomes. In order to determine whether these variations were specific to the virulence, origin, or serovar of a strain, further statistical analyses were performed.

Hierarchical clustering. Data were first scrutinized by the hierarchical clustering analysis developed by Meunier et al. (22). This allowed classification of data according to their global expression profile and grouping of spots with similar
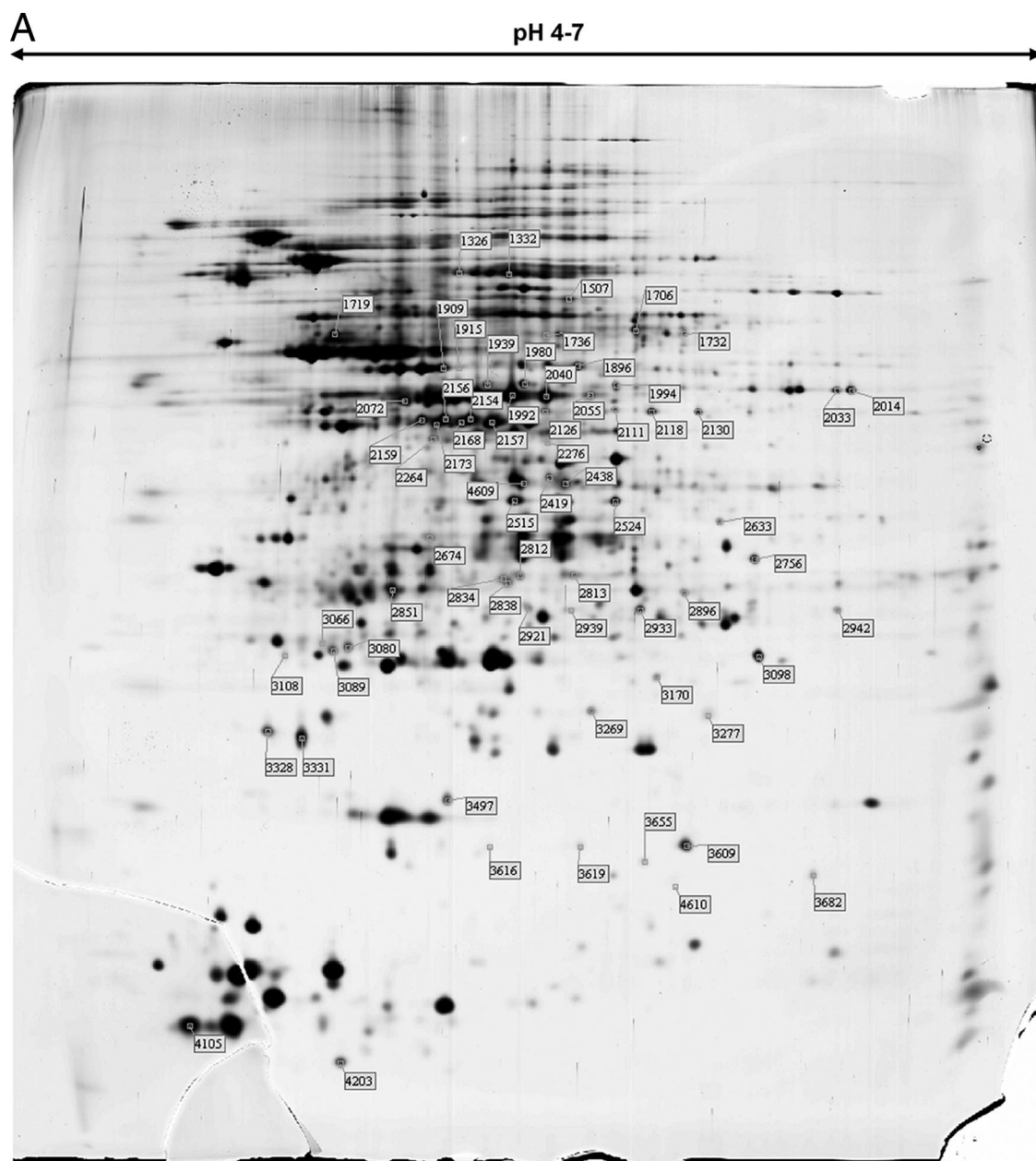
FIG. 1. Reference gels of intracellular (A) and extracellular (B) proteins generated with a mixture of equivalent quantities of proteins from the 12 *L. monocytogenes* strains. Proteins were separated by 2-DE, using pH 3 to 10 nonlinear IPG strips and pH 4 to 7 linear IPG strips for IEF of extracellular and intracellular proteins, respectively. SDS-polyacrylamide gel electrophoresis was performed with a 12.5% acrylamide gel. Labels correspond to the spot numbers listed in Tables 2 and S1 to S3 in the supplemental material.

expression profiles along the samples in order to highlight biomarkers of a specific group. Missing values, due to a real absence of protein spots or a misdetection by the image analysis software, are problematic for clustering algorithms. Thus, only reliably present or absent spots were taken into account (22). As a result, 219 spots for the intracellular fraction and 159 spots for the extracellular fraction were considered reliable. These protein spots constitute the data matrix from which the hierarchical clustering was performed (Fig. 2). First, good intraclass reproducibility could be observed, as all gels of one strain are clustered together. Second, a clear division in two

major clusters can be observed. In the two subproteomes, lineage II serovar 1/2a strains formed a significant cluster clearly distinguished from lineage I serovar 4b and 1/2b strains. These results indicated that the major observed differences between the strains were related to their lineage. The clustering of the protein spots with PermutMatrix permitted the highlighting of spots specific to each lineage. It is also interesting to observe that there are differences between the strains but without any relationship with this factor and that these reflect the biodiversity of *L. monocytogenes*. The specificities of the protein patterns of some strains are more pronounced for one subpro-
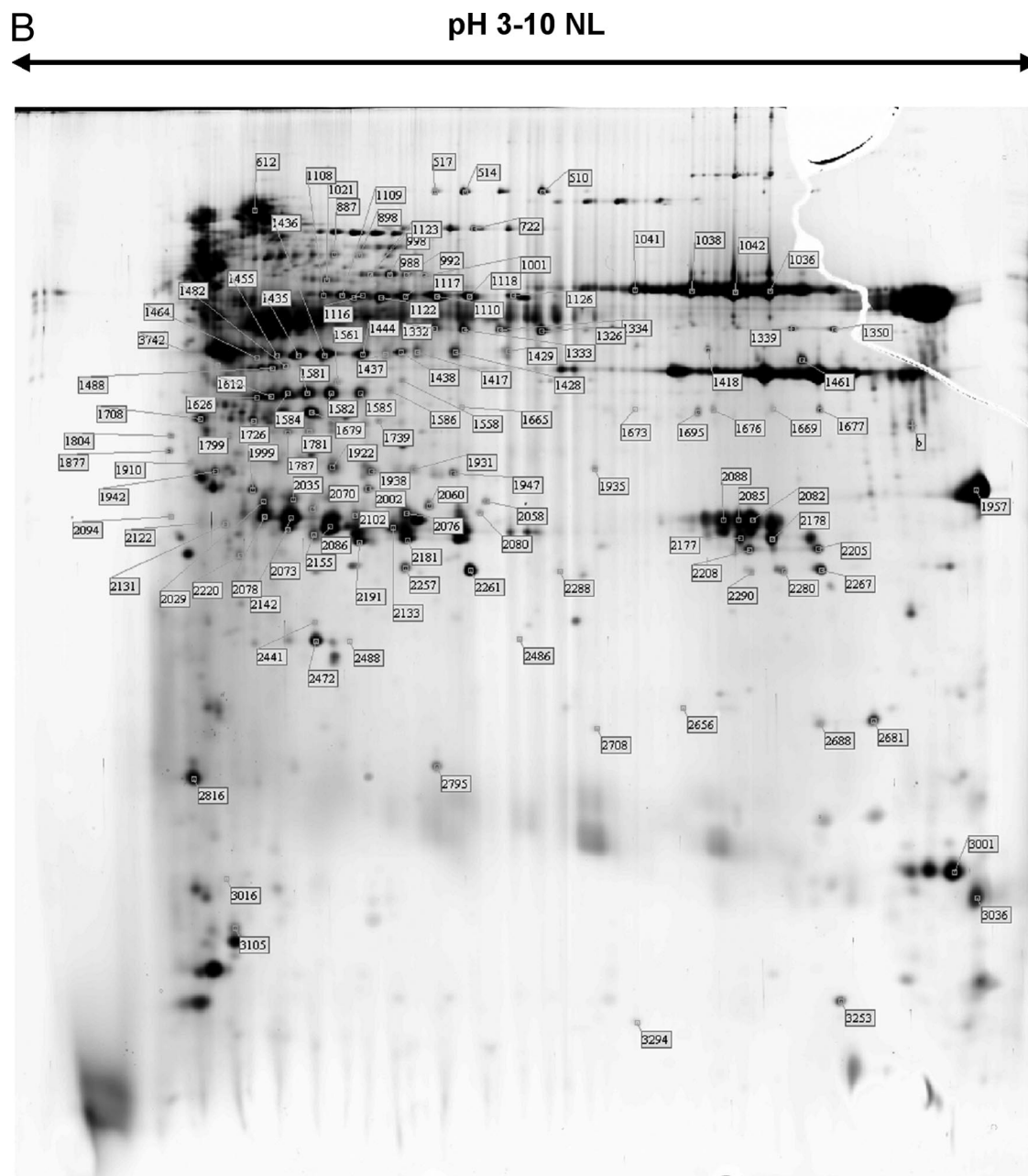
FIG. 1—*Continued.*

teome than the other. For example, strain EGDe (1/2a, clinical strain, virulence A) presents multiple spots, especially in the intracellular proteome, whereas CLIP90602 (1/2b, clinical strain, virulence A) presents marked differences in the extracellular proteome. In fact, the distributions of the strains within the two major clusters are different when comparing the heirarchical clustering analyses of extracellular and intracellular proteomes of different subgroups. Only the subcluster in which the two strains of serovar 4b, i.e., CLIP 93679 and CLIP 93667, are grouped was found in the two subproteomes.

ERV. Data were further analyzed by calculating the ERV for the three factors, i.e., virulence, origin, and serovar, based on

the two matrices containing reliable spots. The higher the ERV, the higher the probability that the value of the spot is different for each of the different factors (virulence, origin, and serovar).

For the virulence, 9 spots from the extracellular proteome and 11 spots from the intracellular proteome had ERV higher than 50% ($P < 10^{-6}$). As the 12 strains presented only two different levels of virulence, a stepwise discriminant analysis was carried out, allowing selection of a subset of independent variables among the two subproteome spots from which a discriminant function was calculated. In this way, an effective linear separation was obtained for the virulence A and B
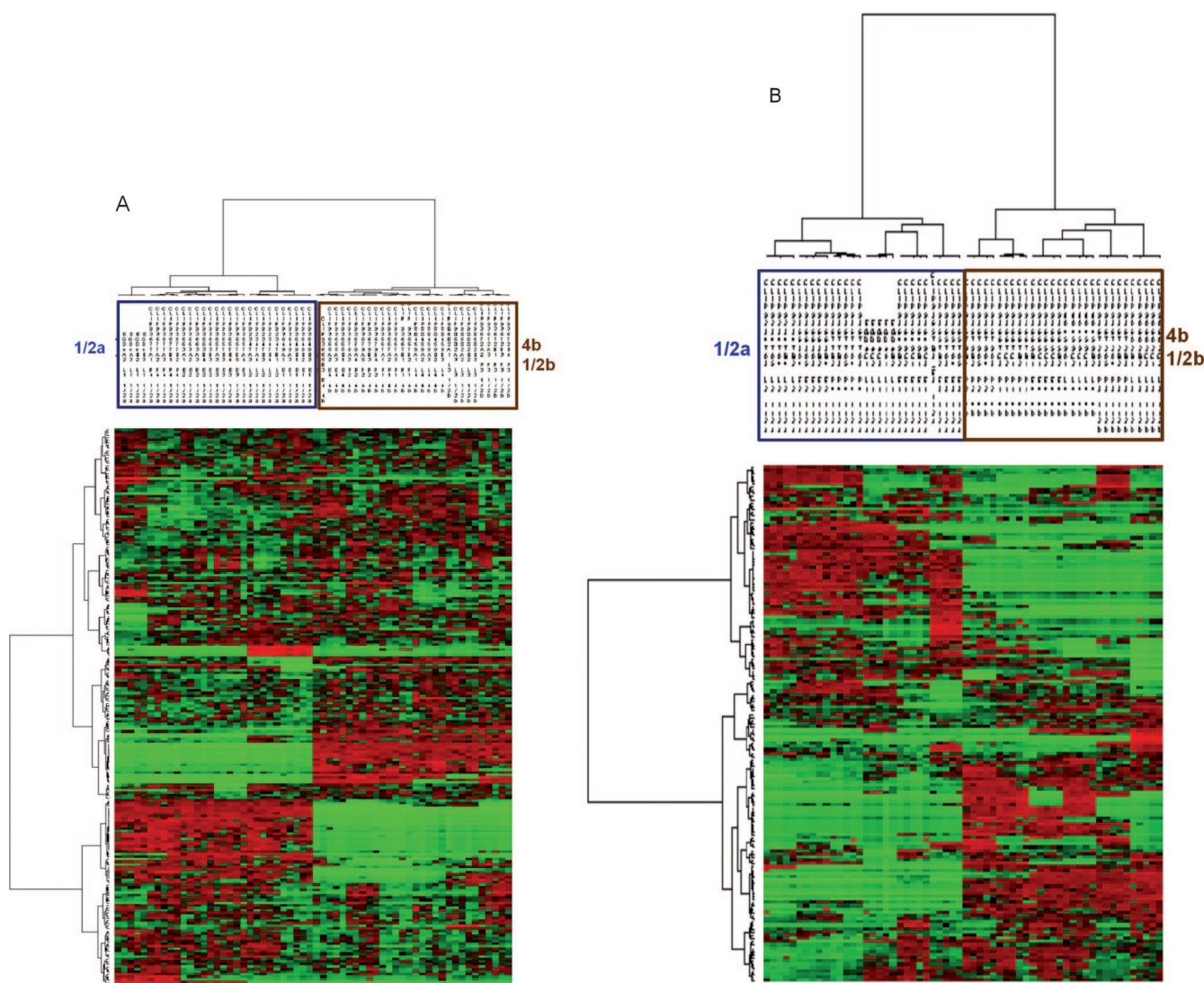
FIG. 2. Hierarchical clustering analysis, carried out with PermutMatrix, from the 60 gels of the 12 *L. monocytogenes* strains and from either the 219 or 159 reliable protein spots of intracellular (A) or extracellular (B) protein fractions, respectively. Increasing intensities of red and green indicate increasing and decreasing protein expression, respectively.

strains with six and seven protein spots from the extracellular and intracellular proteomes, respectively (see Fig. S1 in the supplemental material).

For the origin, only five spots from the extracellular proteome and nine spots from the intracellular proteome had ERV higher than 40% ($P < 10^{-5}$) and no spot had an ERV higher than 59%. As there were more than two classes, an FDA was performed with the most significant spots. This analysis did not allow clear discrimination of strains according to their origin (see Fig. S2 in the supplemental material).

For the two proteomes, the variation of a large number of spots was explained solely by the serovar. Indeed, 69 and 61 spots for the extracellular and intracellular proteomes, respectively, had ERV higher than 50% ($P < 10^{-6}$) (Fig. 3). An FDA performed with spots that had ERV higher than 80% ($P < 10^{-8}$), i.e., 21 and 23 protein spots for the extracellular and intracellular proteomes, respectively, showed a very good dis-

crimination of the strains according to their serovars (100% of well-classified strains after leave-one-out cross validation; data not shown). The discrimination between the three serovars studied was always very clear when only four or five selected protein spots from extracellular or intracellular proteomes, respectively, were used for the FDA (100% of well-classified strains after leave-one-out cross validation; Fig. 3).

Identification of specific protein spots. The previous statistical analyses allowed the highlighting of protein spots differently expressed between the 12 strains, i.e., 199 spots, among which 159 were identified and correspond to 79 proteins. These proteins identified by MALDI-TOF mass spectrometry are listed in Table 2 (serovar specific) and in Tables S1 (serovar specific), S2 (strain specific), and S3 (other differences) in the supplemental material. A good discrimination of the two levels of virulence could be observed when a set of spots was taken into account. However, none of these spots taken individually
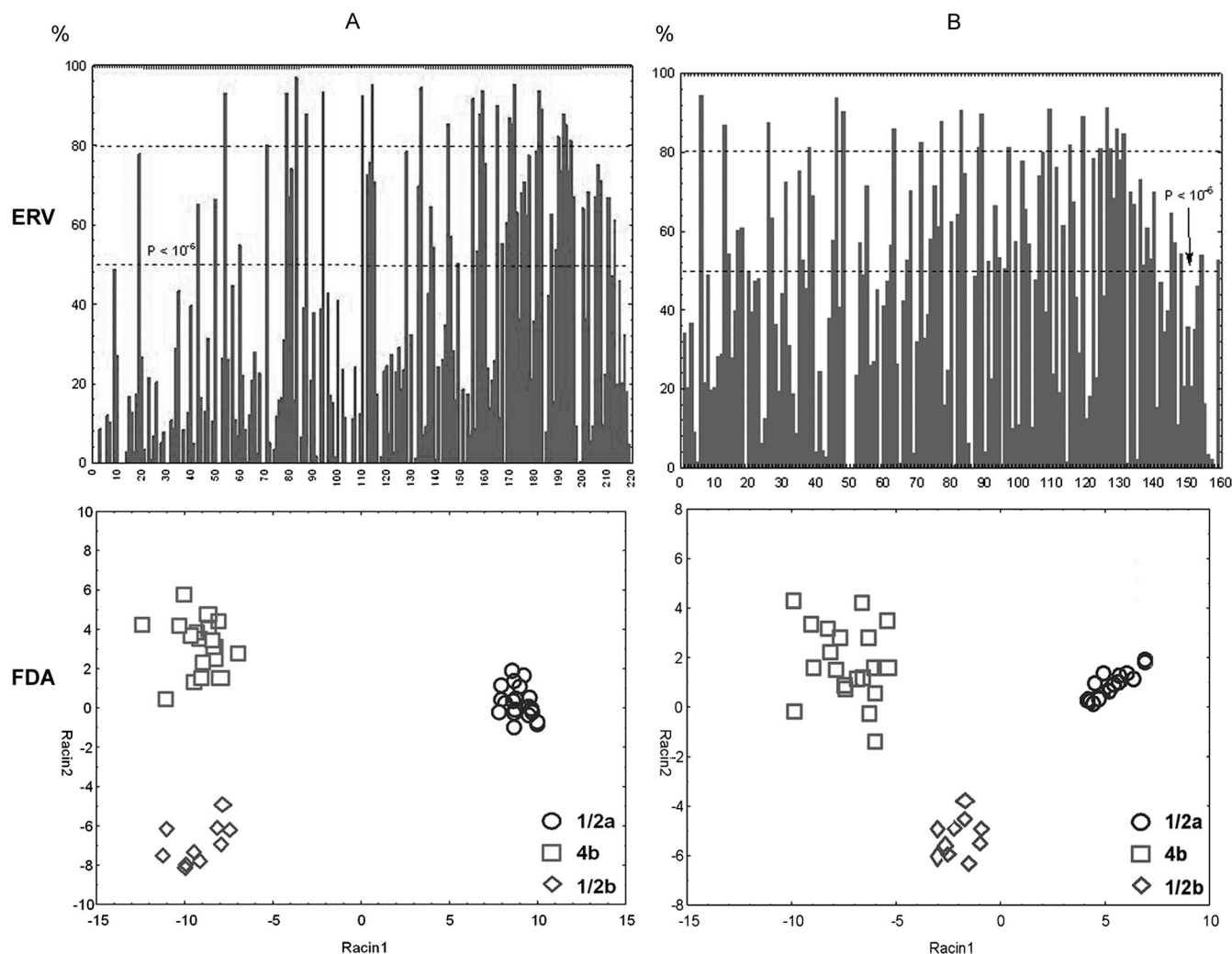
FIG. 3. Representation of the ERV and FDA from the intracellular (A) and extracellular (B) proteome reliability matrix. The ERV (%) for each spot was calculated for the serovar factor. The FDA were carried out with the five most representative spots for the intracellular proteome (translation elongation factor Ts [LMOf2365_1678], the adenylate kinase [LMOf2365_2584], the 2-C-methyl-D-erythritol 4-phosphate cytidylyl-transferase 2 [IspD2], and two unidentified spots) and the four most representative spots for the extracellular proteome (Lmo2522, a peptidase of the M48 family [LMOf2365_2477], a serine protease [LMOf2365_1900], and one unidentified spot). This allowed for very good discrimination of the serovars.

presented a homogeneous level of expression according to virulence group A or B. Nevertheless, it can be noted that three spots of the extracellular proteome were overexpressed in all strains of virulence B and underexpressed in all strains of virulence A, except CLIP92347 (serovar 1/2a, clinical strain, virulence A). Two of these spots (spots 2257 and 2261; Fig. 1B) were identified as Lmo0443, a protein similar to *Bacillus subtilis* transcription regulator LytR. We did not manage to identify the third spot. Statistical analyses of the data matrix did not make apparent some pertinent protein spots, allowing significant discrimination of the strains according to their origin. On the other hand, 26 protein spots (11 extracellular and 15 intracellular proteins) were specifically overexpressed in the six strains of serovar 1/2a (lineage II). An aldoketoreductase (spot 2633; Fig. 1A), a toxic ion resistance protein (spot 1736; Fig. 1A), and an aminopeptidase (spot 2118; Fig. 1A) were identified only in this serovar. Twenty protein spots (9 extracellular

and 11 intracellular proteins) were significantly underexpressed in serovar 1/2a strains. An ATP synthase subunit (spot 3170; Fig. 1A), a secreted phosphoglyceromutase (spot 1021; Fig. 1A), a secreted catalase (spot 1006; Fig. 1B), and secreted proteins of unknown function (spots 2060 and 2816; Fig. 1B) were not detected in serovar 1/2a strains. The other differences were not due to the absence of the protein but to differences in the M_r and pI of the protein between lineage II (serovar 1/2a) and lineage I (serovars 4b and 1/2b). The purine nucleoside phosphorylase DeoD (spot 3080; Fig. 1A), the phosphoglycerate mutase (spot 1326; Fig. 1A), and the pyruvate dehydrogenase (E1 α subunit) PdhA (spots 2014 and 2033; Fig. 1A) were present in the two lineages, but only one out of the two, three out of the four, and two out of the four spots present in serovar 1/2a strains were present in lineage I strains. The nucleotide diphosphate kinase (spot 3655; Fig. 1A) and FtsZ (spot 1719; Fig. 1A) were present in two forms in lineage I but in only one

TABLE 2. Proteins differentially expressed according to serovar[a]

Spot identification	UniProtKB accession no.	Protein description	Expression[b] in serovar:		
			1/2a	1/2b	4b
1736	**Q8Y5T8**	**Toxic ion resistance proteins**	+	−	−
2118	**Q8Y6S2**	**Aminopeptidase**	+	−	−
2633	**Q8Y3X9**	**Aldoreductase/keto reductase**	+	−	−
2168	**Q8Y6M7**	**Translation elongation factor**	+	−	−
2173	**Q8Y6M7**	**Translation elongation factor**	+	−	−
2157	**Q8Y6M7**	**Translation elongation factor**	+	−	−
2942	**Q8Y4P4**	**Similar to *B. subtilis* YhfK protein**	+	−	−
3080	**Q8Y644**	**Purine nucleoside phosphorylase**	+	−	−
1326	**Q8Y4I4**	**Phosphoglyceromutase**	+	−	−
2014	**Q8Y865**	**Pyruvate dehydrogenase (E1 α subunit)**	+	−	−
2033	**Q8Y865**	**Pyruvate dehydrogenase (E1α subunit)**	+	−	−
3066	**Q8Y859**	**Protein of unknown function**	+	−	−
2834	**Q8Y449**	**Adenylate kinase**	+	−	−
2812	**Q8Y971**	**Phosphomethylpyrimidine kinase ThiD**	+	−	−
1507	**Q8Y4C0**	**H+-transporting ATP synthase chain alpha**	+	−	−
2133	Q8Y4C8	LysM domain protein	+	−	−
2142	Q8Y4C8	LysM domain protein	+	−	−
2086	Q8Y4C8	LysM domain protein	+	−	−
1333	Q8Y4E2	Peptidase, M48 family	+	−	−
514	Q8Y4E2	Peptidase, M48 family	+	−	−
1126	Q8Y464	Peptidase, M48 family	+	−	−
1695	Q8Y9I8	PBP	+	−	−
510	Q71WS4	PBP 2A	−	+	+
1334	Q71WS4	Peptidase, M48 family	−	+	+
2708	P21171	Invasion-associated protein p60	−	+	+
2073	Q71WQ6	LysM domain protein	−	+	+
1006	Q71VX5	Catalase	−	+	+
1021	Q71WX0	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	−	+	+
2060	Q721L6	Protein of unknown function	−	+	+
2816	Q71XZ2	Protein of unknown function	−	+	+
2939	**Q71X34**	**Similar to *B. subtilis* YhfK protein**	−	+	+
3655	**Q4EE05**	**Nucleoside diphosphate kinase**	−	+	+
1719	**Q8Y5M5**	**Cell division initiation protein FtsZ**	−	+	+
3089	**Q721A7**	**Protein of unknown function**	−	+	+
2813	**Q722N3**	**Phosphomethylpyrimidine kinase**	−	+	+
2838	**Q71WG7**	**Adenylate kinase**	−	+	+
2156	**Q71Z12**	**Translation elongation factor Ts**	−	+	+
2159	**Q71Z12**	**Translation elongation factor Ts**	−	+	+
2154	**Q71Z12**	**Translation elongation factor Ts**	−	+	+
3277	**Q71Z67**	**General stress protein**	−	+	+
3170	**Q71WP6**	**ATP synthase F1, Δ subunit**	−	+	+
2756	**Q8Y832**	**2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase 2**	+	+	−
2674	**Q8YA72**	**Two-component response regulator**	+	+	−
1350	Q71YE5	Serine protease	−	−	+
1339	Q71YE5	Serine protease	−	−	+
1935	Q721A2	Protein of unknown function	−	−	+
1326	Q71WS4	Peptidase, M48 family	−	−	+
1437	Q8Y3S7	PBP	−	+	−

[a] Spots were identified in the extracellular and intracellular proteomes; for the latter, characters are in boldface. Spots were all identified from sequenced *L. monocytogenes* strains, i.e., EGDe (serovar 1/2a), H7858 (serovar 4b), and F2365 (serovar 4b).

[b] +, overexpressed or present; −, underexpressed or absent.

form in lineage II. Also, a zinc metallopeptidase (spots 1333, 514, 1126, 1695, and 1334; Fig. 1B), a LysM domain protein (spots 2133, 2142, 2086, and 2073; Fig. 1B), a penicillin-binding protein (spots 1695 and 510; Fig. 1B), and a protein similar to the *B. subtilis* YhfK protein (spots 2942 and 2939; Fig. 1A) were present in the two lineages but at positions clearly different on the 2-DE gels in the same number or in different numbers of spots. Less spots were differently expressed in strains of serovars 4b and 1/2b than in strains of serovar 1/2a. Indeed, only two protein spots identified as a serine protease (spots 1339 and 1350; Fig. 1B) were present in the four strains of serovar 4b; these spots were absent

in the strains of the other two serovars. Two spots, identified as the putative 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase 2 (spot 2756; Fig. 1A) and a two-component response regulator (spot 2674; Fig. 1A), were specifically underexpressed in serovar 4b strains. Only one protein, corresponding to a PBP (spot 1437; Fig. 1B), was overexpressed in serovar 1/2b.

DISCUSSION

This analysis showed a large biodiversity among *L. monocytogenes* isolates. Indeed, none of the strains investigated had

the same protein pattern, and a lot of strain-specific spots have been highlighted. The extracellular proteomes revealed more-pronounced differences in the expression profiles than the intracellular proteomes. From previous genomic studies of 93 *L. monocytogenes* strains, it appeared that the strains possessed all known virulence genes (10). From the present investigation, however, some differences could be observed at the proteomic level for some virulence factors. Indeed, PlcB (spots 2177, 2208, 2267, 2280, and 2290; Fig. 1B), and InlC (spots 2181, 2102, 2191, and 2076; Fig. 1B) were present in multiple spots with different pIs and M_rs. The presence or absence of these spots differed among the strains. Moreover, and contrary to what was found for the 11 other strains, *L. monocytogenes* strain CLIP90602 (clinical strain, serovar 1/2b, virulence A) did not express, or expressed at a much lower level, virulence factors ActA, LLO, PlcA, and PlcB. A lower expression of virulence genes in some strains responsible for listeriosis had been previously reported (23, 30, 38). While it can be argued that expression of virulence factors might be induced upon host cell infection, the two other clinical strains investigated here, i.e., CLIP92347 and CLIP80459, expressed these genes. However, the possibility that environmental conditions and stimuli required for expression of listerial virulence factors (as well as the expression levels) could vary from one strain to another cannot be ruled out.

Although the two levels of virulence, A and B, could be discriminated when a set of spots was taken into account, none of these spots was a marker of the virulence group when taken individually. However, it was noted that two spots (spots 2257 and 2261; Fig. 1B), identified as a protein of the LytR/CpsA/Psr family and found in the extracellular proteome, were overexpressed in all strains of virulence B and underexpressed in all strains of virulence A, except CLIP92347 (serovar 1/2a, clinical strain, virulence A). This protein family clearly exhibits cell wall maintenance properties via autolysin regulation (7), which further suggests that the difference of expression levels reflects distinct cell wall activities in the two virulence groups under the present experimental conditions.

Statistical analyses of results failed to discriminate the investigated strains according to their origins (i.e., from human listeriosis cases, asymptomatic carriage, or food plant environment). It must be stressed that, despite the great care taken in the choice of the strains according to these three origins, this categorization is not so clear-cut. The possibility that, in the case of asymptomatic carriage, some patients could have developed listeriosis later on cannot be completely excluded. The development of listeriosis depends on the immune state and the ingested dose. Concerning food plant isolates, such *L. monocytogenes* strains could have been previously involved in human listeriosis. Such information obviously has an impact on the statistical analysis of data but unfortunately could not have been taken into consideration. In addition, the culture conditions of bacterial strains in a laboratory under optimal growth parameters (pH 7.0, 37°C, high nutrient concentrations) are far different from the conditions encountered by bacteria in their natural environment and might have masked the specificity of the ecological niche. To highlight such differences, further experimental investigations would be necessary, such as comparison of strains cultured in conditions that could mimic to some extent those encountered in the host (low-pH environ-ment of stomach, antimicrobial environment of mucosal surfaces, presence of bile and enzymes in the gastric fluid, etc.) or in the food plant environment (low temperature, high concentration of NaCl). Nonetheless, statistical analysis permitted a good discrimination of the strains according to their serovars and hierarchical clustering showed that the investigated strains could be discriminated into two clusters, i.e., (i) cluster I including strains of serovar 1/2a and (ii) cluster II, including serovars 4b and 1/2b. Antigenic properties were one of the first criteria used to classify *L. monocytogenes* strains, following a scheme developed by Seeliger and Höhne describing 13 distinct serovars (32). Based on the extracellular and intracellular proteome analyses, our results corroborate divisions previously established (5) and further revealed a strong correlation between the extracellular and intracellular protein contents and serovars. This result is of great significance, as the presence of genes does not reflect the expression of an encoded protein, which in the end is the functional entity. Regulation of expression at different levels, i.e., transcriptional or translational, by, for example, rendering a promoter nonfunctional or repression of transcription/translation, could explain the observed differences in these experimental conditions. It must be stressed that the absence of some proteins in some of the strains tested can be due to the detection limit of the proteomic technique and should be confirmed by further investigations using enzyme-linked immunosorbent assay, for example. Beyond the expression level of proteins, this investigation also provided more-subtle information. Indeed, the absence of a spot on a gel at a particular location did not necessarily reflect the absence of the protein. Some orthologues with different M_rs, pIs, and/or posttranslational modifications migrated at different positions on the 2-DE gels. It further appeared that the presence of such protein spots was serovar or lineage dependent; as numerous studies have shown the importance of protein phosphorylation for protein activities in *L. monocytogenes* (1), such specific expression of some spots might mirror differences in protein functionality (2, 8, 19, 31).

The statistical analyses according to the serovar factor resulted in identifying a total of 26 protein spots specific to lineage II (or serovar 1/2a), including 11 extracellular and 15 intracellular spots, and 20 protein spots specific to lineage I (serovars 1/2b and 4b), including 9 extracellular and 11 intracellular proteins. One of the proteins present only in the lineage I strain is catalase (spot 1006; Fig. 1B), primarily predicted to be cytoplasmic but found in the extracellular milieu. The localization of a protein in two subcellular compartments has been previously observed in gram-positive bacteria, and this catalase is indeed predicted to be extracellular by SecretomeP, with a score of 0.58 (3). In *B. subtilis*, extracellular catalase is essential to ensure complete protection against oxidative stress (24). Although numerous spots allowed discrimination of serovar 1/2a strains (lineage II) from strains of the two other serovars (lineage I), only a few protein spots were specific to serovar 4b strains or serovar 1/2b strains. From analysis of the extracellular proteome, two protein spots identified as a serine protease (spots 1339 and 1350; Fig. 1B) were present in the four strains of serovar 4b but absent from the other eight strains. By demonstrating protein expression, the present proteomic analysis corroborates previous microarray data where the gene encoding this serine protease was shown

as specific to strains of serovar 4b (10). Serine proteases are known to be important for stress resistance and virulence of *L. monocytogenes*. For example, HtrA is essential for survival after exposure to many types of environmental and cellular stresses and necessary for virulence in mice (34, 40, 41). ClpP of *L. monocytogenes* acts as a serine protease and prevents the accumulation of altered proteins that might be toxic for the bacteria under stress conditions (12, 13). Moreover, in the absence of ClpP, the secretion of functional LLO is reduced (13). Only one protein was specific to strains of serovar 1/2b, i.e., PBP Lmo2754 (spot 1437; Fig. 1B). PBPs are transpeptidases involved in different aspects of the cell wall synthesis in bacteria and contribute in various degrees to β-lactam resistance, cell morphology, and virulence potential (18).

Beyond differential protein expression, correlated with the serovars and phylogenetic lineages of the listeria strains studied here, this investigation highlighted the large biodiversity of *L. monocytogenes* isolates based on analyses of two subproteomes, namely, the extracellular and intracellular proteomes. This differential regulation of protein expression between the different strains may have an impact on clinical behavior and niche adaptation. This investigation gives an initial view of the protein expression of *L. monocytogenes* species in these two subproteomes, keeping in mind that it was performed within a defined set of experimental conditions and that the conclusions drawn hold true only in this particular situation. In order to generalize them, further studies under various growth conditions (different media, temperatures, pHs, salt concentrations, host cells, etc.) should be carried out and would certainly result in variation of the patterns of these subproteomes. Similar statistical analyses should then be applied to confirm whether or not these proteomic patterns still correlate with serovars. While this study was based on the most common serovars involved in human listeriosis cases, a similar approach should be widened to other serovars as well as avirulent strains to have a clearer overview of the biodiversity and specificity of the expression profiles for *L. monocytogenes* strains. Ultimately, this would allow for the targeting of protein functions of interest or important protein markers and thus the development of strategies to face listerial risks.

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